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We have hypothesized that intra-tumoral injection of immature DCs should result in acquisition of tumor antigens at the site of injection followed by migration to lymph node and spleen. Here we have used adenoviral vectors, able to efficiently infect both murine and human DC, to deliver IL-12, IL-18, and GM/CSF and the costimulatory molecules B7.1 and CD40L, either alone or in combination, to bone marrow-derived DC (BmDC). We showed that the genetic modification of DCs to overexpress IL-12, GM/CSF, and CD40L significantly enhanced their ability to stimulate a systemic immune response following intra-tumoral injection. In addition, we have used adenoviral gene transfer of members of the TNF family able to stimulate apoptosis, FasL and TRAIL, to render DC direct, potent mediators of tumor cell apoptosis. We demonstrate that the genetic modification of DCs by adenoviral infection to overexpress IL-12, IL-18, and GM/CSF significantly enhanced their ability to stimulate a systemic immune response in the murine breast tumor TS/A following intra-tumoral injection. Furthermore, expression of FasL and TRAIL on DC resulted in a stronger anti-tumor response following intra-tumoral delivery. The anti-tumor effect of different genetically modified-DC was mediated, in part, by the induction of specific CTL activity against the tumor. The genetic modification DC to express one or more immuno-stimulatory molecules was able to augment NK cells and CTL cytolytic activity and IFN-gamma production for subsequent immune anti-tumor responses locally as well as systemically. We currently are examining the optimal combination of genes in order to induce the most effective anti-tumor response following intra-tumoral infection of the genetically modified DC. Our research is expected to lead Phase I clinical trial to assess the feasibility and efficacy of treating and preventing breast cancer by intra-tumor injection of genetically modified DCs.

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INTRODUCTION

We have hypothesized that intra-tumoral injection of immature dendritic cells (DCs) should result in acquisition of tumor antigens at the site of injection followed by migration to lymph node and spleen. Indeed, we have demonstrated previously that flourescently labeled DCs are able to migrate to lymph nodes and spleen following intra-tumoral injection and stimulate a tumor specific T cell response (1). Systemic delivery of BmDC promotes prophylactic and therapeutic anti-tumor immunity (2-6). Furthermore, immature DC injected intra-tumorally (IT) acquire tumor-associated antigen (TAA) in situ and then process and present the captured antigens in lymphoid organs. Presentation of these antigen naïve T cells should efficiently initiate a tumor specific immune response (7-9). The IT injected DCs are able to migrate to lymph nodes where they can present TAA acquired at the tumor site. Here we have used adenoviral vectors, able to efficiently infect both murine and human DC, to deliver interleukin-12 (IL-12), interleukin-18 (IL-18), and granulocytes and macrophages colony stimulating factor (GM/CSF) and the costimulatory molecules CD80 (or B7.1) and CD40 ligand (CD40L), either alone or in combination, to bone marrow-derived DC (BmDC). We showed that the genetic modification of DCs to overexpress IL-12, GM/CSF, and CD40L significantly enhanced their ability to stimulate a systemic immune response following intra-tumoral injection. In addition, we have used adenoviral gene transfer of members of the tumor necrosis factor (TNF) family able to stimulate apoptosis, FasL and TNF-related apoptosis-inducing ligand (TRAIL), to render DC direct, potent mediators of tumor cell apoptosis. We demonstrate that the genetic modification of DCs by adenoviral infection to overexpress IL-12, IL-18, and GM/CSF significantly enhanced their ability to stimulate a systemic immune response in the murine breast tumor TS/A following intra-tumoral injection. Furthermore, expression of FasL and TRAIL on DC resulted in a stronger anti-tumor response following intra-tumoral delivery. The anti-tumor effect of different genetically modified-DC was mediated, in part, by the induction of specific cytotoxic T lymphocytes (CTL) activity against the tumor. The genetic modification DC to express one or more immuno-stimulatory molecules was able to augment natural killer (NK) cells and CTL cytolytic activity and interferon-gamma (IFN-γ) production for subsequent immune anti-tumor responses locally as well as systemically. We currently are examining the optimal combination of genes in order to induce the most effective anti-tumor response following intra-tumoral infection of the genetically modified DC. Our research is expected to lead Phase I clinical trial to assess the feasibility and efficacy of treating and preventing breast cancer by intra-tumor injection of genetically modified DCs.

BODY

Task 4. Optimization of Dendritic cell function for anti-tumor immunity, Month 13-18:

a. Screening genes for achievement of enhancing the activity on DC.
b. Analysis changing of surface markers with transgene expression on DC by flow cytometry.
c. Test of cytokines production from genetically-modified dendritic cells.
d. Migration assay of DCs for specific chemokines.

Bone marrow-derived DC was generated from 6-8 week old age female Balb/C mouse as described previously (10). Bone marrow from the tibia and femur of the mouse was collected and passed through a nylon mesh. Erythrocytes was lysed with NH_4Cl buffer, and lymphocytes depleted using a cocktail of antibodies (RA3-3A1/6.1, anti-B220; 2.43, anti-Lyt2; GK1.5, anti-L3T4; all from ATCC, Rockville, MD) and rabbit complement on day 0. Remaining cells were cultured for 24 hr in complete media (CM). The non-adherent cells were placed in fresh CM containing 1000 U/ml of rmGM/CSF and rmIL-4 on day 1. Cells were cultured 4 more days and harvested for transduction on day 4.

For infection, $1X10^6$ DC was collected in the round-bottomed tube. The appropriate viruses (50 MOI) including Ad-GM.CSF, Ad-IL-10, Ad-IL-12, Ad-B7.1, Ad-CD40L, and Ad-lacZ, in 1 ml of serum free media were added into the tube and mixed with cells. After incubation for 2 hr in room temperature, CM was added and incubated for 20 hr. On day 5, the cultured media of infected DC was replaced with CM containing rmGM/CSF and rmIL-4. The DC was remained for 2 days and then stained with antibodies for phenotypic analysis, or *in vitro* or *in vivo* experiments.

For phenotypic analysis of bone marrow-derived DC (day 7), FITC- or PE-conjugated monoclonal antibodies recognizing murine cell surface molecules (CD11c, CD80, CD86, major histocompartibility class (MHC) I and II, and CD40) were used. After incubation with antibodies for 30 min. on ice, cells were washed with phosphate buffered saline (PBS) and analyzed on a FACStar using Cellquest FACS analysis software (Becton Dickinson).

DC derived from mouse bone marrow exhibited the veiled dendrite morphology typical for DC and displayed a characteristic set of DC surface marker (Table 1). The genetically modified DCs expressed high levels of the MHC class I and II molecules, the costimulatory molecules B7.1 and B7.2, ICAM-1 adhesion molecule, and integrin CD11c. Infection of DC with Ad-IL-12, Ad-GM/CSF, and Ad-CD40L at a 50 MOI resulted in a marked increase in levels of expression of B7.1, B7.2 and MHC class I molecules. For example, 52.6% of CD40L-, 46% of IL-12-, and 47% of Ad-GM/CSF-transduced DC expressed CD86 molecules in comparison with 24.6% and 26.9% in non-transduced and Ad-psi5 control virus-transduced cells, respectively.

Cytokine production from the genetically modified DCs and migration ability of DCs for specific chemokines were measured as described in the last annual report.

Task 5. Evaluation of anti-tumor effect with DCs administration in breast tumor, Month19-24:

a. Intra-tumoral injection of genetically-modified dendritic cells on tumor mice.
b. Measurement of tumor growth with DC administration.

TS/A cells, an aggressive and poorly immunogenic tumor line derived from a Balb/C breast adenocarcinoma , were maintained in RPMI1640 media. Female 6-8 week-old Balb/C mice were injected intra-dermally with 2×10^5 TS/A cells on day 0. On day 7, palpable tumor was detected on the site of flank injected with the cells. On day 7, a million of transduced DC with Ad-GM/CSF, Ad-IL-12, Ad-CD40L, Ad-B7.1, or Ad-eGFP, or non-transduced DC were injected intra-tumorally. Tumor growth was determined every 3 days by measuring the long and short diameter of tumor mass with

Burnier calipers. The treatment of genetically engineered DC expressing the cytokines such as GM/CSF, IL-12, IL-10 (Fig 1), surface molecules such as B7.1, CD40L (Fig 2), and TNF family such as TRAIL and 4-1BBL (Fig 3) significantly suppressed growth of the established breast tumor. Furthermore, Ad-CD40L induced IL-12 secretion from the transduced DC and showed efficient anti-tumor immune responses (data not shown). Moreover, combined injection with DC/GM-CSF+CD40L and DC/IL-12+B7.1 yielded efficient growth inhibition of the breast tumor (Fig 4). After an injection of DC/CD40L on day 7, established TS/A tumor gradually decreased and completely disappeared in six out of ten treated mice (Fig 2A). Re-challenged TS/A tumor was rejected in the CD40L-treated mice, while the challenged CT26 cells formed tumor on the other side of flank (data not shown).

KEY RESEARCH ACCOMPLISHMENTS

1. Generation of Bone marrow-derived dendritic cell from Balb/C mouse
2. Purification of adenoviruses by CsCl gradients
3. Transduction of DCs with adenoviruses
4. Phenotypic analysis of the transduced using flow cytometry
5. Induction of TS/A breast tumor in Balb/C mouse model
6. Injection of the transduced DCs intra-tumor site in established tumor
7. Measurement of tumor size
8. Evaluation of anti-tumor effect with DCs injection
9. Re-challenge of TS/A or non-specific syngenic tumor in the cured mouse

REPORTABLE OUTCOMES

1. Generation of adenoviruses including Ad/GM/CSF, Ad/IL-12, Ad/IL-10, Ad/CD40L. Ad/B7.1, Ad/TRAIL, Ad/ 4-1BBL, and Ad/eGFP in large scale
2. Abstracts for the 5th annual meeting of American society of gene therapy (appended)

CONCLUSIONS

1. Bone marrow-derived dendritic cells (BmDC) were infected 50 MOI of adenoviruses encoding potent therapeutic genes. Surface molecule genes were delivered to 75 ~ 98 % of total DC. A million of DC which was infected with Ad-cytokines secreted 15 ~ 40 ng/ml of cytokines in the culture media for 48 hours.

2. Each dendritic cell could be infected with two or more different type of adenoviruses. Even though adenoviral infection caused slight higher levels of MHC and co-stimulatory molecules on DC surface, double infection might not increase the level of surface molecules

3. Intra-tumoral injection of DC/CD40L and DC/IL-12 showed the significant suppression of growth in TS/A breast tumor model. Moreover, combined injection with

DC/GM-CSF+CD40L and DC/IL-12+B7.1 yielded efficient growth inhibition of the breast tumor.

5. Splenocytes from the DC/CD40L- or DC/IL-12- injected mouse produced higher level of IFN-g with stimulation of the irradiated TS/A tumor cell. In contrast, Th2 type cytokines such as IL-4 and IL-10 were maintained in low level in regardless of type or number of genes, or DC injection (data not shown).

6. We demonstrated that administration of the genetically engineered DC showed the anti-tumor effect on the established breast tumor model. We suggested the possible treatment of tumor with combined gene delivery mediated by DC.

7. We currently are examining the optimal combination of genes in order to induce the most effective anti-tumor response following intra-tumoral infection of the genetically modified DC. The results of these experiments should lead to a Phase I clinical trial to assess the feasibility and efficacy of treating and preventing breast cancer by intra-tumor injection of genetically modified DCs.

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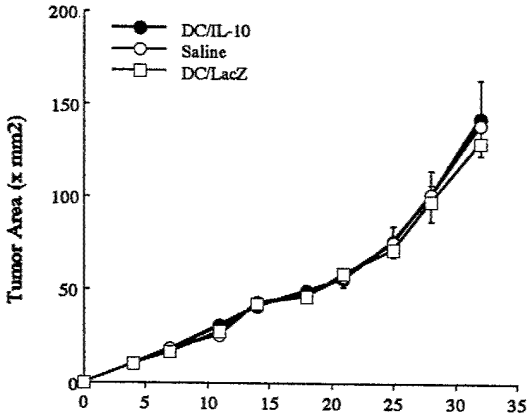
Table 1. Phenotyping of DCs transduced with adenoviruses.

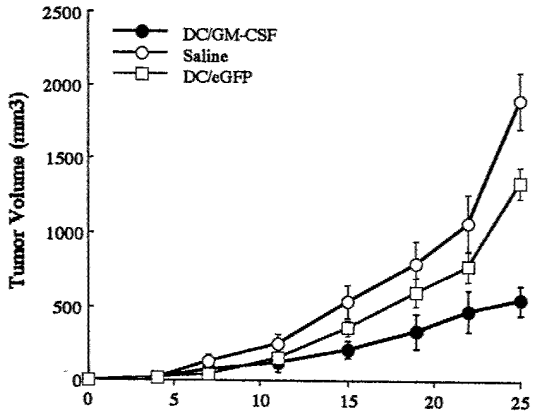
DCs	CD11c	CD80	CD86	Iad	CD40
Control	76%	46%	20%	80	26%
Ad/lacZ	74%	56%	27%	78	32%
Ad/IL-10	84%	74%	37%	80	28%
Ad/IL-12	82%	72%	46%	82	33%
Ad/GM.CSF	80%	74%	47%	84	31%
Ad/CD40L	78%	64%	53%	87	52%

Figure 1. Administration of DC expressing Cytokines in the established TS/A Breast Tumor of Balb/C Mouse

DC/mIL-10 in TS/A



mGM/CSF



mIL-12

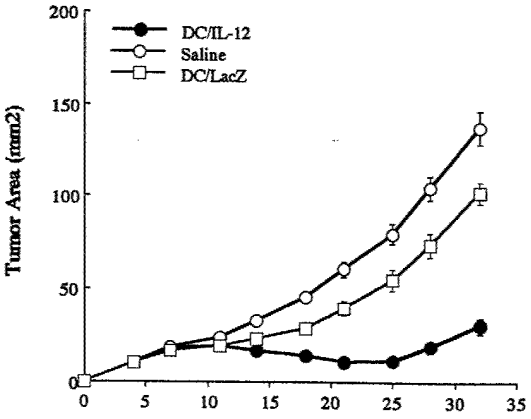


Figure 2. Administration of DC expressing surface molecules in the established TS/A Breast Tumor of Balb/C Mouse

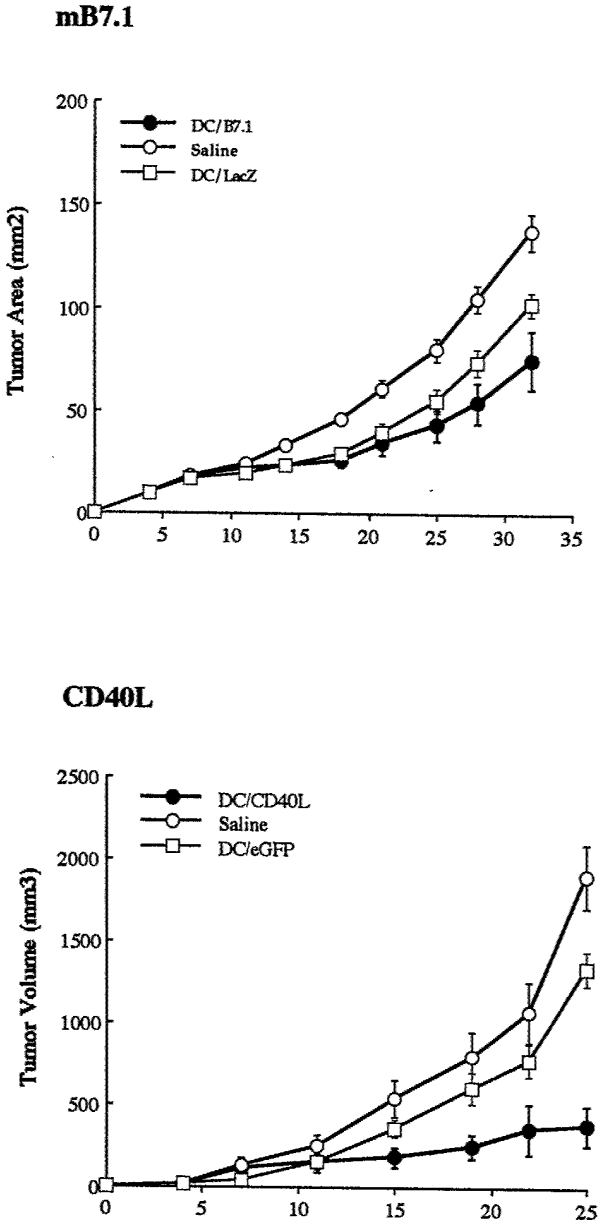
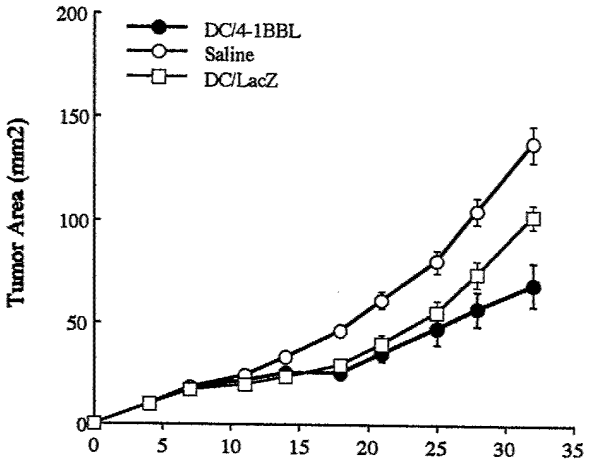


Figure 3. Administration of DC expressing TNF family into the established TS/A Breast Tumor in Mouse Model

4-1BBL



TRAIL

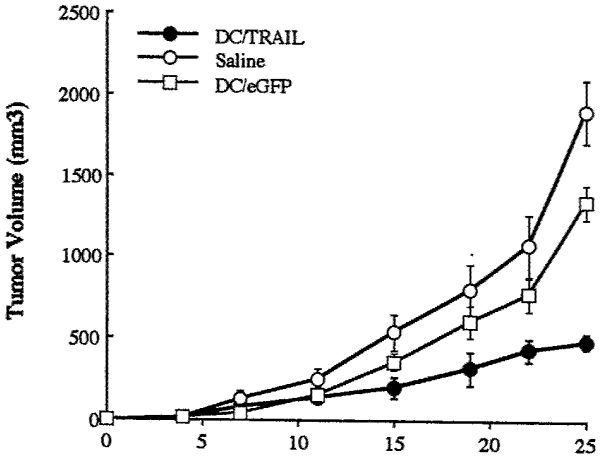
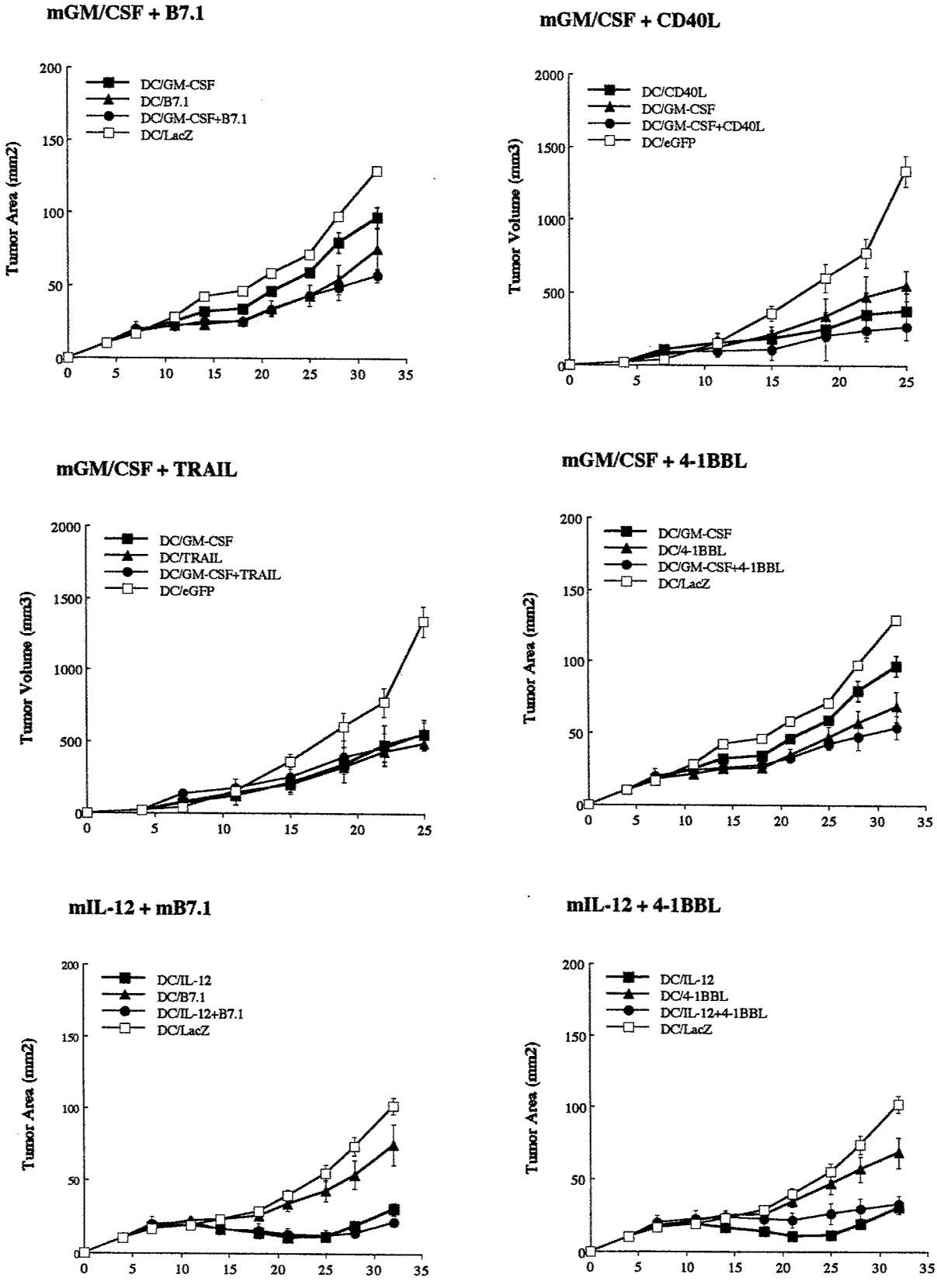


Figure 4. Combined Gene Delivery using DC to Treat the established TS/A Breast Tumor in Mouse Model



Combined Immunotherapy of a Murine Mammary Tumor using Genetically Modified Dendritic Cells. Seon Hee Kim, Zoya R. Yurkovetsky and Paul D. Robbins, Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA

We have hypothesized that intra-tumoral injection of immature DCs should result in acquisition of tumor antigens at the site of injection followed by migration to lymph node and spleen. Indeed, we have demonstrated previously that flourescently labeled DCs are able to migrate to lymph nodes and spleen following intra-tumoral injection and stimulate a tumor specific T cell response. Here we have used adenoviral vectors, able to efficiently infect both murine and human DC, to deliver IL-12, IL-18, and GM/CSF and the costimulatory molecules B7.1 and CD40L, either alone or in combination, to bone marrow-derived DC (BmDC). We showed that the genetic modification of DCs to overexpress IL-12, GM/CSF, and CD40L significantly enhanced their ability to stimulate a systemic immune response following intra-tumoral injection. In addition, we have used adenoviral gene transfer of members of the TNF family able to stimulate apoptosis, FasL and TRAIL, to render DC direct, potent mediators of tumor cell apoptosis. We demonstrate that the genetic modification of DCs by adenoviral infection to overexpress IL-12, IL-18, and GM/CSF significantly enhanced their ability to stimulate a systemic immune response in the murine breast tumor TS/A following intra-tumoral injection. Furthermore, expression of FasL and TRAIL on DC resulted in a stronger anti-tumor response following intra-tumoral delivery. The anti-tumor effect of different genetically modified-DC was mediated, in part, by the induction of specific CTL activity against the tumor. The genetic modification DC to express one or more immuno-stimulatory molecules was able to augment NK cells and CTL cytolytic activity and IFN-gamma production for subsequent immune anti-tumor responses locally as well as systemically. We currently are examining the optimal combination of genes in order to induce the most effective anti-tumor response following intra-tumoral infection of the genetically modified DC. Our research is expected to lead Phase I clinical trial to assess the feasibility and efficacy of treating and preventing breast cancer by intra-tumor injection of genetically modified DCs.